Flow-Through pH-Stat Method for Lipase Activity

A new method for lipase activity which combines the simplicity and rapidity of continuous pH-stat methods with the flexibility in choice of conditions (pH, temperature, etc.) of manual methods is described. The lipase-catalyzed hydrolysis of olive oil and titration of fatty acid products can be carried out separately in two small continuously stirred reactors in series. The response is linear with enzyme dilution.

KEY WORDS: enzyme activity; triglyceride hydrolysis; initial rate assay; lipolysis; lipolytic.

Lipases (EC 3.1.1.3) are a ubiquitous class of enzymes which catalyze the hydrolysis of fatty acids from their glycerol esters. They are industrially important in the formation of both desirable and undesirable flavors in milk and dairy products (1,2). More recently, interest has arisen in their application to processing of bulk oleochemicals (3,4). Methods for detection and determination of lipase have been recently reviewed (5). Many of these require the use of unnatural substrates (tributyrin, radioactive, and chromogenic esters) and therefore cannot be used to study the conditions of industrially important reactions. Others require tedious extraction and separation techniques (GC, HPLC, and TLC). The standard method for fatty acids (AOAC 28.029) (6) is titration in hot ethanol to a phenolphthalein endpoint. This method was adapted for lipase activity measurement (7). Other investigators have used a glass pH electrode instead of pH indicators (8,9). Development of the automatic buret and pH-stat have led to methods in which continuous titration is carried out simultaneously with the enzymatic hydrolysis reaction in the same vessel (10–14). These methods are rapid and simple to carry out, but suffer from two disadvantages. First, the enzyme concentration in the reaction mixture is gradually diluted by the addition of titrant. This problem can be reduced, but not eliminated, by increasing the concentration of the basic titrant solution. Second, the pH at which the reaction is carried out must be a compromise between the optimum for the enzyme and the significantly higher pH required for complete titration of fatty acids. As a result, complete titration is not achieved and measurement of optimum pH with these methods does not reflect the true properties of the enzyme. Described here is a new method which eliminates these two problems while retaining and improving upon most advantages of the continuous pH-stat methods. In this method, enzyme and substrate are pumped into a stirred emulsion reactor where they react and flow to a second stirred vessel for titration of the fatty acid products. Thus, the enzymatic and acid–base reactions are carried out separately.

MATERIALS AND METHODS

Chemicals used included olive oil from Felippo Berio, oleic acid (food grade, Baker), lipase M (from *Candida,* 30 units/mg according to the seller, Enzeco), 0.1 N sodium

hydroxide standard solution (Fisher), sodium azide (Fisher), and Triton X-100 (Rohm and Haas).[1] Equipment included Manostat 10-channel cassette pump, Metrohm E526 titrator with 655 Dosimat (automatic buret), and Omega Model 49 temperature controller. Equipment was arranged as shown in Fig. 1. Technicon Solvaflex pump tubes were used for olive oil (0.025 in. i.d.), enzyme solution (0.025 in. i.d.), and waste (0.11 in i.d.). Silicone tubing (0.032 in. i.d. × 0.163 in. o.d.) was used for pumping ethanol. All other tubes were Teflon spaghetti tubing (0.030 in. i.d. × 0.062 in. o.d.). Technical-grade ethanol (95%) was titrated to pH 10.4 before use. Olive oil was stored in brown glass at room temperature under nitrogen. Olive oil was not purified or emulsified before use. Enzyme was dissolved in 0.02 M phosphate buffer containing 0.02% sodium azide and 0.01% Triton X-100 and the solution was clarified by centrifugation. Enzyme solution and olive oil were fed to the continuous emulsion reactor at 0.28 and 0.20 ml/min, respectively. The continuous emulsion reactor consisted of a 150 × 20-mm culture tube stoppered with an inverted No. 1 silicone rubber stopper penetrated by three Teflon tubes which served as inlets for olive oil and enzyme solutions and an outlet for emulsion. The stopper was inserted nearly to the bottom of the tube which also contained a $\frac{3}{8} \times \frac{5}{8}$-in. magnetic stir bar. The reactor was maintained at constant temperature (37°C) by a glass water bath which rested on a magnetic stirrer. The height of the bath through which the feed tubes passed brought the reactants up to reaction temperature before they entered the reactor. The olive oil tube protruded from the bottom of the inverted stopper, almost touching the stir bar, to prevent the lower-density oil from channeling across the top of the reactor. Stirring speed was kept as high as possible without uncoupling of the magnets, so that the finest emulsion possible was maintained. The exit tube was recessed to facilitate removal of air from the reactor. Residence time was 5.1 min and conversion less than 5%. The pH did not change significantly from inlet to outlet.

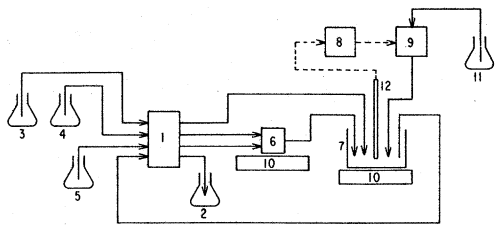

FIG. 1. Schematic diagram of apparatus for flow-through pH-stat method. Key: (1) Peristaltic pump; (2) waste receptacle; (3) ethanol reservoir; (4) olive oil reservoir; (5) lipase sample solution; (6) continuous-emulsion reactor; (7) titration vessel; (8) pH controller; (9) automatic buret; (10) magnetic stirrer; (11) 0.1 N NaOH reservoir; (12) pH electrode (combination).

Products of the reaction flowed to the titration vessel where the pH was maintained at 10.4 by the pH-stat, which displayed the cumulative volume of 0.1 N NaOH added. The titration vessel consisted of a glass vial (30 mm diameter × 52 mm high). The waste pump tube could pump up to several milliliters per minute, and its inlet was positioned to maintain a constant liquid level in the titration vessel. The alcohol pump tube pumped about 0.2 ml/min of ethanol to the titration vessel. The combination of high pH and ethanol completely inactivated the enzyme in the titration vessel. From start-up or from switching to a different enzyme solution, the system took 20–30 min to equilibrate. Then the pH-stat volume display was reset to zero and its value was recorded every 2 min for 10 min. Linear regression on the data gave the rate (μmol/min) of acid leaving the emulsion reactor. A second data set was

[1] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

taken after a 10-min wait. The first two sets of data usually agreed within a few percent, but if they did not agree within 5%, additional data sets were taken, each after a 10-min wait, until equilibration was indicated by the agreement within 5% of two consecutive data sets.

The activity of enzyme in the reactor in International Units (IU) was simply the rate of titratable acid leaving the emulsion reactor minus the rate entering. The rates of acid entering (called blanks) were calculated separately for the olive oil and enzyme. For the enzyme, a 4.0-ml sample was mixed with 4.0 ml of ethanol in a clean vial and titrated to pH 10.4. The acid concentration (μmol/ml enzyme solution) multiplied by the measured enzyme flow rate (ml/min) gave the enzyme blank (μmol/min). For olive oil, 4.0 ml of ethanol was added to a weighed sample (about 4 g) of oil and the mixture was titrated to pH 10.4. The acid concentration (μmol/g) multiplied by the measured flow rate (ml/min) and the density (0.91 g/ml) gave the oil blank (μmol/min). The flow rates were measured under actual reaction conditions using a stopwatch and graduated cylinders as feed reservoirs. The flow rates and pH-meter calibration were checked about once a week. The endpoint of 10.4 was chosen as giving the best agreement with the molecular weight of pure oleic acid in batch titrations. Agreement was then checked by pumping known concentrations of oleic acid in olive oil through the system with water. The pH-stat could handle up to 20% oleic acid (more than 100 μmol/min) with less than 2% deviation from theoretical, provided stirring in the titration vessel was sufficient. In activity measurements, rates did not usually exceed 20 μmol/min.

The activity of the lipase sample solution (IU/ml or units/ml if other than standard conditions) was calculated as the activity of enzyme in the reactor divided by the volume of enzyme solution in the reactor. The total volume of the emulsion reactor including the entire emulsion exit tube was measured by filling it with acid of known concentration, which was then flushed into a beaker with distilled water (pH 10.4) and titrated to pH 10.4. This total reactor volume was multiplied by the ratio of enzyme flow rate to total flow rate to find the volume of enzyme solution in the reactor:

$$2.44 \text{ ml} \cdot \frac{0.28 \text{ ml/min}}{(0.20 + 0.28 \text{ ml/min})} = 1.42 \text{ ml.} \quad [1]$$

A sample calculation of lipase activity follows:

Raw data:

Time (min)	0	2:00	4:00	6:00	8:00	10:00
Volume (ml)	0	0.19	0.37	0.54	0.72	0.91

Linear regression on raw data ($r^2 = 0.9997$):

Rate of acid leaving reactor

$$= 9.01 \text{ micromoles/min}$$

Enzyme blank

$$= \left(\frac{43 \ \mu\text{mol}}{4 \text{ ml}}\right)\left(0.28 \frac{\text{ml}}{\text{min}}\right) = 3.01 \frac{\mu\text{mol}}{\text{min}}$$

Oil blank

$$= \left(\frac{17 \ \mu\text{mol}}{4 \text{ g}}\right)\left(0.91 \frac{\text{g}}{\text{ml}}\right)\left(0.20 \frac{\text{ml}}{\text{min}}\right) = 0.77 \frac{\mu\text{mol}}{\text{min}}$$

Activity in reactor

$$= 9.01 - 3.01 - 0.77 = 5.23 \text{ IU}$$

$$\text{Activity in solution} = \frac{5.23}{1.42} = 3.68 \text{ IU/ml.}$$

RESULTS AND DISCUSSION

The data in Fig. 2 for Enzeco lipase show that the measured activity is directly propor-

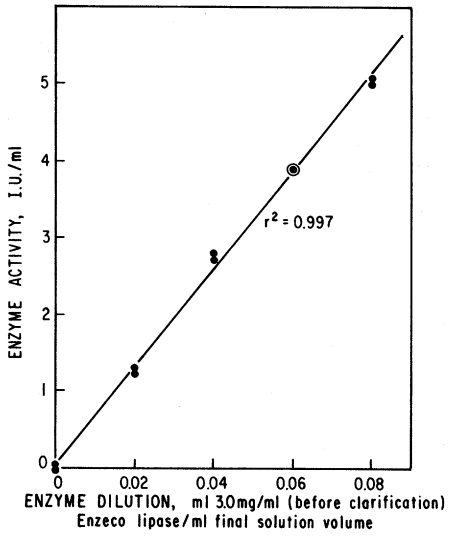

FIG. 2. Activity of different dilutions of a 3.0-mg/ml (before clarification) solution of Enzeco lipase. Stock enzyme solution and dilutions were made with 0.02 M phosphate buffer, pH 6.8, containing 0.01% Triton X-100 and 0.02% sodium azide.

tional to enzyme dilution at low enzyme concentration. From these data, standard statistics of the linear regression were used to calculate an estimated standard deviation of 0.1 IU/ml. Therefore, the method's precision is ±2% at 5 IU/ml with a sensitivity of 0.2 IU/ml. It is well known that lipase is fully active only at an oil–water interface and that under certain conditions the activity is proportional to interfacial area (15). Unfortunately, the interfacial area of a stirred emulsion is difficult to measure or control. At high enzyme concentration, the measured activity is less than predicted by extrapolating the straight line in Fig. 2. This is probably due to an interfacial surface area limitation.

This is the first continuous pH-stat method in which the conditions for the lipase-catalyzed reaction and the titration of fatty acid products are separately controlled. Thus, the best conditions for titration of fatty acid products can be used in spite of their extremely inhibitory effect on most lipases, while the lipase-catalyzed reaction is carried out under optimum conditions. Therefore, this method should yield higher lipase activities than previously published pH-stat methods. The advantage over previous pH-stat methods is most obvious with lipases that are optimally active at low pH. The benefit of the pH-stat is primarily one of automation. With proper interfacing, a computer could do the linear regression automatically, or slopes could be taken from a strip chart recorder. In this flow-through pH-stat method, pipetting is reduced and the assay can be carried out repeatably by an unskilled worker with minimum training. At the same time, none of the flexibility of batch methods is lost. The ratio of oil to enzyme solution in the reactor can be varied by changing the pump tube diameters, and the residence time can be varied by adjusting the pump speed. Some experimentation with these parameters is required to minimize experimental error and equilibration time while maximizing activity. Different lipases may have different requirements in this regard. The effects of emulsifiers, activators, inhibitors, pH, temperature, ionic strength, product concentration, different substrates, and different lipases can all be easily determined with this system. It is particularly suitable for thermal deactivation studies because the enzyme feed reservoir can be held at constant temperature and its decreasing activity monitored continuously.

In summary, the method presented here combines the simplicity, ease of operation, and rapidity of previously described pH-stat methods with the flexibility in choice of reaction conditions of manual methods. As such, it represents an important contribution to the study of lipases, enzymes of increasing economic significance.

REFERENCES

1. Seitz, E. W. (1974) *J. Amer. Oil Chem. Soc.* **51,** 12–16.

2. Seitz, E. W. (1976) *Biotechnol. Bioeng.* **18,** 889–938.
3. Werdelmann, B. W., and Schmid, R. D. (1982) *Fette Seifen Anstrichmittel* **84,** 436–443.
4. Linfield, W. M., Barauskas, R. A., Sivieri, L., Serota, S., and Stevenson, R. W. (1984) *J. Amer. Oil Chem. Soc.* **61,** 191–195.
5. Jensen, R. G. (1983) *Lipids* **18,** 650–657.
6. Horowitz, W. (1980) *in* Official Methods of Analysis of the AOAC, 13th Ed., p. 441, AOAC, Washington, D.C.
7. Bier, M. (1955) *in* Methods in Enzymology (Colowick, S. P., and Kaplan, N. O., eds.), Vol. 1, p. 627, Academic Press, New York.
8. Iwai, M., Tsujisaka, Y., and Fukumoto, J. (1964) *J. Gen. Appl. Microbiol.* **10,** 13–22.
9. Moskowitz, G. J., Shen, T., West, I. R., Cassaigne, R., and Feldmann, L. I. (1977) *J. Dairy Sci.* **60,** 1260–1265.
10. Parry, R. M., Chandan, R. C., and Shahani, K. M. (1966) *J. Dairy Sci.* **49,** 356–360.
11. San Clemente, C. L., and Vadehra, D. V. (1967) *Appl. Microbiol.* **15,** 110–113.
12. Schifreen, R. S., and Carr, P. W. (1979) *Anal. Lett.* **12,** 47–69.
13. Peled, N., and Krenz, M. C. (1981) *Anal. Biochem.* **112,** 219–222.
14. Rick, V. W. (1969) *Z. Klin. Chem. Klin. Biochem.* **7,** 530–539.
15. Benzonana, G., and Desnuelle, P. (1965) *Biochem. Biophys. Acta* **105,** 121–136.